

Human T-Cell Leukemia Virus Type 1 Tax Protein Down-Regulates Pre-T-Cell Receptor Alpha Gene Transcription in Human Immature Thymocytes[∇]

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The human pre-T-cell receptor alpha (TCR α ; pT α) gene encodes a polypeptide which associates with the TCR β chain and CD3 molecules to form the pre-TCR complex. The surface expression of the pre-TCR is pT α dependent, and signaling through this complex triggers an early $\alpha\beta$ T-cell developmental checkpoint inside the thymus, known as β -selection. E2A transcription factors, which are involved at multiple stages of T-cell development, regulate the transcription of the pT α gene. Here we show that the regulatory protein Tax of the human T-cell leukemia virus type 1 (HTLV-1) efficiently suppresses the E47-mediated activation of the pT α promoter. Furthermore, we report that in Tax lentivirally transduced human MOLT-4 T cells, which constitutively express the pT α gene, the amount of pT α transcripts decreases. Such a decrease is not observed in MOLT-4 cells transduced by a vector encoding the Tax mutant K88A, which is unable to interact with p300. These data underline that Tax inhibits pT α transcription by recruiting this coactivator. Finally, we show that the expression of Tax in human immature thymocytes results in a decrease of pT α gene transcription but does not modify the level of E47 transcripts. These observations indicate that Tax, by silencing E proteins, down-regulates pT α gene transcription during early thymocyte development. They further provide evidence that Tax can interfere with an important checkpoint during T-cell differentiation in the thymus.

The early intrathymic developmental pathway of human $\alpha\beta$ T lymphocytes is regulated by an immature form of the T-cell receptor (TCR) complex (pre-TCR) consisting of the rearranged TCR β chain and the invariant surrogate pre-TCR α (pT α) chain in association with molecules from the CD3 signaling complex, upstream of the critical β -selection checkpoint. When assembled at the cell membrane, the pre-TCR complex transmits an essential survival signal for the development of T cells and is thought to play roles in allelic exclusion of TCR β . The transcription of the pT α gene has been shown to be regulated through the basic helix-loop-helix (bHLH) transcription factors, also called E proteins (27), which bind specific sequences (E-box; CANNTG) contained in the regulatory sequences of this gene (30, 40). Up-regulation of pT α transcription coincides with irreversible T-cell lineage commitment in the thymus and is mainly observed in immature thymocytes (5, 6, 43). After the completion of the β -selection step, Id (inhibitors of DNA binding) proteins down-regulate pT α gene expression. These proteins possess the HLH domain, conferring to them the ability to heterodimerize with E proteins, but lack the basic DNA binding motif. Thus, Id proteins block the transcriptional activity of the E proteins by sequestering them away from the E-boxes (3). Finally, E proteins, like other bHLH proteins, have been shown to bind to the p300/CREB binding protein (CBP) coactivators (2, 16, 34).

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia (ATL), an aggressive and fatal leukemia that develops in less than 5% of HTLV-1-infected individuals (31). Among the viral regulatory proteins encoded by the provirus, the transcriptional transactivator Tax protein is thought to be the primary effector of T-cell activation. Indeed, in addition to its role in activating proviral transcription, Tax up-regulates cellular gene expression mainly by activating a variety of transcription factors that interact with promoters of target genes (46). Tax has been shown to interact with transcription factors, such as cyclic AMP response element-binding protein/activating transcription factor (CREB/ATF), NF- κ B, and p67 serum-responsive factor. Tax is also known to down-modulate the transcription of cellular genes mediated by bHLH proteins by preventing the interactions of these proteins with p300/CBP coactivators, thus leading to the inhibited activity of these transcription factors (33, 38).

HTLV-1 can productively infect human hematopoietic CD34⁺ progenitor cells as well as human immature thymocytes (13, 25). It was also demonstrated that reconstitution of T lymphopoiesis with HTLV-1-infected CD34⁺ cells in severe combined immunodeficient mice engrafted with human thymus and liver tissues (SCID-hu) resulted in the perturbation of thymopoiesis and an aberrant display of thymocyte subpopulations (13). Furthermore, it has been shown that HTLV-1 Tax-transduced hematopoietic (CD34⁺) cells isolated from human fetal liver have a reduced capacity to proliferate, as evidenced by the induction of cell cycle arrest and the concomitant suppression of multilineage hematopoiesis (41, 42).

Here, we provide evidence that Tax down-modulates pT α gene expression. By transient transfections together with a

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reporter luciferase gene under the control of the regulatory sequences of the pT α gene, we show that Tax efficiently suppresses the E47-mediated activation of the pT α gene. Furthermore, we have used lentivirus-mediated gene transfer to express Tax in human lymphoblastoid pT α -expressing MOLT-4 T cells and nucleofection in human immature thymocytes. We observe that the expression of Tax correlates with a decrease of the amount of pT α transcripts but does not interfere with the transcription of E47. Taken together, these observations indicate that Tax, by decreasing the transcriptional activity of E2A proteins, inhibits pT α gene expression, which might perturb early thymocyte development.

MATERIALS AND METHODS

Cells and plasmids. Normal human thymocytes were obtained from thymus fragments removed during cardiac surgery of patients aged 1 week to 2 years. Thymic lobes were gently minced in RPMI 1640 (Invitrogen) containing 2% fetal calf serum (Dutscher, France) and antibiotics (100 IU/ml penicillin, 50 μ g/ml streptomycin). Cells were isolated by Ficoll density gradient centrifugation (Ficoll-Paque Plus; StemCell Technologies Inc., Vancouver, BC, Canada). Thymocytes recovered from the Ficoll interface were enriched for immature cells using the EasySep CD3-positive selection cocktail to deplete CD3⁺ cells followed by a negative selection using human CD4⁺ T-cell enrichment cocktail (StemCell Technologies Inc., Vancouver, BC, Canada) according to the manufacturer's instructions.

The immature cell line MOLT-4 (American Type Culture Collection) was grown in RPMI supplemented with 10% fetal calf serum, 5 mM L-glutamine, and HEK-293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum and antibiotics.

The pT α reporter element EP180, together with the two mutants EP180 Δ E and EmP180 containing deletion or base substitutions of the E-boxes, respectively, were a generous gift from A. Takeuchi (40). The Tax expression plasmid, pCMV-Tax, containing the *tax* coding sequence under the control of the cytomegalovirus promoter, was a generous gift from W. C. Greene. The pCMV-E47 and -p300 expression vectors were previously described (33).

Production of retroviral and lentiviral vectors, transduction, and nucleofection. One retroviral vector, Id3-IRES-GFP, and three lentiviral vectors, pHR-TaxYFP, pHR-TaxK88AYFP, and pHR-eYFP, were used in the present study. The retroviral vector (generous gift of H. Spits) contains the Id3 coding sequence linked to a downstream internal ribosomal entry site (IRES) and the enhanced green fluorescent protein (GFP) sequence (4). The lentiviral vectors pHR-TaxYFP and pHR-TaxK88AYFP encode either an HTLV-1 Tax cDNA fused to the enhanced yellow fluorescent protein (YFP) sequence or a TaxK88A-enhanced YFP sequence, respectively, under the control of the cytomegalovirus promoter (8, 10). The lentiviral pHR-eYFP vector contains only the enhanced YFP sequence. The viruses were produced by transient transfection, using the calcium phosphate DNA precipitation method, of 293T cells seeded at 5×10^6 in 100-mm dishes the previous day, with the three plasmids; the transfer vectors pHR-eYFP, pHR-TaxYFP, pHR-TaxK88AYFP, or Id3-IRES-GFP (6 μ g); a human immunodeficiency virus-derived packaging plasmid, pCMV Δ R8.91 (28), or murine leukemia virus-derived packaging plasmid pCMV gag/pol (4 μ g), respectively; and an envelope expression plasmid, pCMV-VSV-G (1 μ g). After 5 to 6 h, medium was changed and cells were incubated in complete DMEM. Vector supernatants were collected 36 h later. Vector supernatants were cleared by low-speed centrifugation, filtered through 0.45- μ m filters (Millipore), and ultracentrifuged at 20,000 rpm for 2 h at 4°C. The vector-enriched pellet was resuspended in DMEM. Vector stocks titers were determined by infecting 293T cells, and 3 days later, they were analyzed for YFP and GFP expression by flow cytometry. Titers ranged from 10^5 to 10^6 transducing units per ml. MOLT-4 cells were transduced at a multiplicity of infection (MOI) ranging from 2 to 5. Seven days later, MOLT-4 cells were sorted on the basis of GFP or YFP expression by fluorescence-activated cell sorting (FACS) on a FACSVantage (Becton Dickinson). Immature thymocytes, cultured overnight in the presence of 10 ng/ml interleukin-7 (R&D, Abingdon, United Kingdom) and 10 ng/ml stem cell factor (Peprotech, Rocky Hill, NJ), were transfected either with 2 μ g of pHR-TaxYFP or pHR-YFP by nucleofection (human CD34 cell nucleofector kit; Amaxa, Köln, Germany) according to the manufacturer's instructions. Twenty-four hours later, YFP⁻ and YFP⁺ cells were sorted from both cultures as indicated above.

Transient-transfection and luciferase assays. HeLa cells (3×10^5 cells) were transfected with 1 μ g of pT α promoter-firefly luciferase constructs and 10 ng of thymidine kinase *Renilla* luciferase (tk-luc) by the calcium phosphate precipitation technique (7). Reporter gene analysis was performed 18 h after transfection by using a Dual-Luciferase reporter assay system (Promega). The luciferase activity associated with each construct was normalized on the basis of tk-luc activity.

Immunoprecipitation and Western blot analysis. Detection of the Tax protein in MOLT-4 cells was performed by immunoprecipitation followed by Western blotting. Briefly, 4 days posttransduction, 10^6 cells were lysed at 4°C in RIPA buffer (0.5 ml of 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate) containing 0.25 mM phenylmethylsulfonyl fluoride and Complete protease inhibitors (Roche Diagnostics). The lysates were then cleared for 30 min at 14,000 \times g. Cleared lysates were preincubated with 20 μ l of protein G Plus/protein A-agarose (Calbiochem) for 4 h; after centrifugation, supernatants were incubated overnight at 4°C with an anti-Tax monoclonal antibody (Covablab, Lyon, France). After adding 50 μ l of protein G Plus/protein A-agarose, the mixture was incubated for 30 min at +4°C. The immunoprecipitated proteins were washed five times with 0.5 ml of RIPA (without SDS and sodium deoxycholate) at 4°C and resuspended in 20 μ l of Laemmli buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.2% bromophenol blue, 100 mM dithiothreitol) and then subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE). For Western blotting, transfected HeLa cells were lysed in Laemmli buffer, and equal amounts of samples were subjected to SDS-PAGE and transferred to nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech) with a semidry blotting device. Membranes were blocked in phosphate-buffered saline containing 10% nonfat milk, 0.1% Tween 20. They were then probed with the appropriate following antibodies: rabbit polyclonal anti-Tax antibody (generous gift from W. C. Greene), mouse monoclonal antiactin (clone AC-40; Sigma), and mouse monoclonal anti-E47 (sc-416; Santa Cruz) and thereafter with an anti-mouse (Dako) or -rabbit (Immunotech, Marseille, France) immunoglobulin G-horseradish peroxidase-conjugated goat antibody. Blots were then developed using an enhanced chemiluminescence detection system (Renaissance; NEN Life Science Products).

FACS analysis. Thymocytes were incubated for 30 min with the following antibodies conjugated with the fluorochromes phycoerythrin (PE), phycoerythrin-cyanin 5 (PC5), or fluorescein isothiocyanate (FITC): anti-CD4-PC5 or anti-CD8-FITC (Immunotech, Marseille, France), anti-CD34-PE (Pharmingen, San Jose, CA), and the control immunoglobulin G1 (IgG1) isotypes mouse-PC5, -FITC, or -PE (Pharmingen, San Jose, CA). They were analyzed by fluorescence cytometry on a FACSCalibur (Becton Dickinson).

RT-PCR and quantitative real-time PCR. Cytoplasmic RNAs were extracted from 10^5 MOLT-4 cells using the RNeasy purification kit (QIAGEN). Total RNA was extracted from thymocytes using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Before reverse transcription, RNAs were first treated with 10 U of RNase-free DNase I (QIAGEN) for 30 min at 27°C and then for 10 min at 60°C. This RNA sample was then reverse transcribed at 42°C for 1 h in a total volume of 20 μ l reaction buffer (50 mM Tris, pH 8.3, 30 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol) containing 100 U of SuperScriptII RNase H⁻ reverse transcriptase (RT; Invitrogen), 100 pmol of oligo(dT)₁₂₋₁₈ (Invitrogen), and 20 U of RNase inhibitor (Rnasin; Promega). A reaction without RT was performed in parallel to serve as control for the absence of DNA contamination. PCR assays were done in 25- μ l reaction volumes using the appropriate diluted amounts of cDNA template, 2 mM MgCl₂, 0.25 mM each deoxynucleoside triphosphate, 10 pmol of each primer, and 1 U of *Taq* DNA polymerase (Invitrogen) in 1 \times buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 5% dimethyl sulfoxide). Reaction conditions were as follows: 5-min denaturing step at 95°C followed by 30 to 40 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C. PCR products were separated on a 2% agarose gel stained with ethidium bromide. The Tax, actin, pT α , and E47 primers that were used were the following: Tax forward, 5'-TGTTTGGAGACTGTGTACAAGGCG-3', and Tax reverse, 5'-GTTGTATGAGTGATTGGCGGGGTA-3', generating a 237-bp fragment; β -actin forward, 5'-TGAGCTGCGTGTGGCTCC-3', and β -actin reverse, 5'-GGCATGGGGGAGGGCATAACC-3', generating a 247-bp fragment; pT α forward, 5'-CATCCTGGGAGCCTTTGGT-3', and pT α reverse, 5'-CCG GTGTCCCTGAGAG-3', generating a 135-bp fragment; E47 forward, 5'-A GTACGGACGAGGTGCTGTC-3', and E47 reverse, 5'-GCTTTGTCCGACT GTAGGTG-3', generating a 158-bp fragment.

Real-time quantitative PCR (qPCR) detection of pT α expression was performed on a LightCycler system (Roche) using the LightCycler-FastStart DNA Master SYBR green I kit. In brief, reactions were performed in a 20- μ l final volume and contained 1.5 μ l of FastStart reaction mix SYBR green, 4 mM

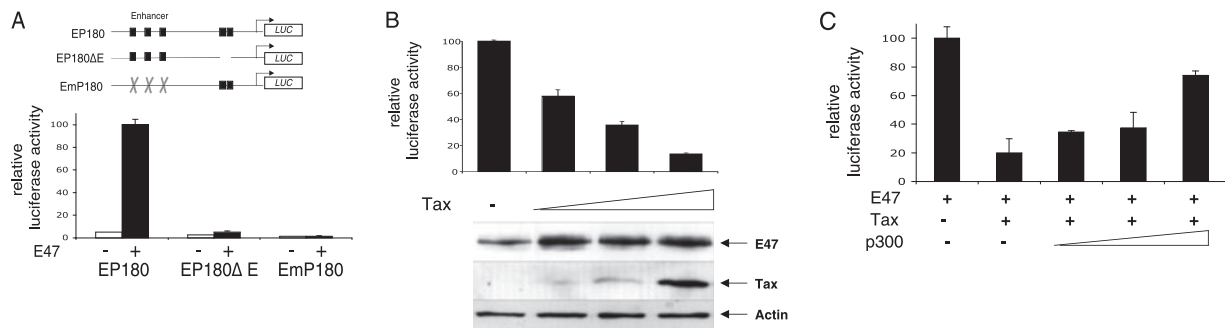


FIG. 1. Activation of the regulatory sequences of the pT α gene in the presence of E47 and/or Tax. (A) E47 enhances the activity of the enhancer and promoter sequences of the pT α gene. Above, schematic representation of the reporter plasmids encoding the luciferase gene under the control of enhancer and promoter sequences of the pT α gene containing either intact (■) E-boxes (EP180), or deleted E-boxes in the promoter (EP180ΔE), or base-substituted E-boxes in the enhancer (EmP180). HeLa cells were transfected with 1 μ g of EP180, EP180ΔE, or EmP180 with (black bars) or without (white bars) 0.75 μ g of pCMV-E47. Luciferase activities were normalized with tk-luc activities and are presented relative to those in cells transfected with EP180 and pCMV-E47 constructs. The values are those obtained in triplicate, from one representative experiment (out of three experiments) \pm the standard deviation. (B) The HTLV-1 Tax protein represses E47-mediated transcriptional activation of the regulatory sequences of the pT α gene. HeLa cells were transfected with 1 μ g of EP180 with 0.75 μ g of pCMV-E47 and increasing concentrations (50 to 500 ng) of pCMV-Tax plasmid. Luciferase activities were normalized with tk-luc activities and are presented relative to those in cells transfected with EP180 and pCMV-E47 constructs, in the absence of Tax. Eighteen hours after transfection, the cells were harvested and an aliquot of each cell extract was resolved on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The same membrane was probed successively with a polyclonal anti-Tax or monoclonal anti-E47 and anti-actin antibodies. (C) The p300 coactivator releases Tax-induced inhibition of E47-dependent transcription of the pT α gene. HeLa cells were cotransfected with 1 μ g of EP180, 0.75 μ g of pCMV-E47, and 0.5 μ g of pCMV-Tax, and with increasing amounts of the p300 expression plasmid (50 to 250 ng). Luciferase activities were normalized with tk-luc activities and are presented relative to those in cells transfected with EP180 and pCMV-E47 constructs, in the absence of Tax and p300.

MgCl₂, 0.5 μ M primers, and 2 μ l of cDNA. The reaction conditions were 95°C for 8 min and then 45 cycles of 10 s at 95°C, 5 s at 61°C, and 10 s at 72°C. Calibration curves were derived by running two- to threefold serial dilutions of cDNA obtained from a positive cell line. Cellular cDNA samples were run at several dilutions. Controls included RT⁻ RNA samples and water. The threshold cycle values (*C_t*) were used to plot the calibration curve. Standard curves had a coefficient of variation of at least 0.98. The copy numbers, normalized to the human actin values measured in a separate tube, are the results of at least two determinations made in duplicate.

RESULTS

Tax represses E47-induced activation of pT α promoter activity. The restricted expression of the pT α gene depends on promoter and enhancer elements containing E-boxes, potential binding sites for bHLH E2A transcription factors, such as E12 and E47 proteins. Three indicator plasmids, in which the luciferase reporter gene was fused to the enhancer and promoter sequences of the pT α gene, containing either wild-type (EP180) or mutated (EmP180) or deleted (EP180ΔE) E-boxes, were used in transient-cotransfection assays. To lower the background activities of the enhancer and promoter sequences, these assays were performed in epithelial HeLa cells in which T-cell-specific regulatory elements are normally inactive (32). An increase in luciferase activity was observed in cells cotransfected with the pCMV-E47 and the EP180 plasmids (Fig. 1A). In cotransfection experiments performed with EP180ΔE (from which the two E-boxes in the promoter region were deleted) or EmP180 (in which base substitutions were introduced in the three E-box sites in the enhancer region), luciferase activity could not be detected in the presence of E47. These observations confirm previous results demonstrating that E-box elements in both the enhancer and promoter regions are regulating the activation of the pT α gene (30, 40).

We next evaluated the effect of Tax on the activity of the pT α promoter in HeLa cells cotransfected with the pCMV-

E47 plasmid and the EP180 reporter construct, together with increasing amounts of a Tax expression plasmid (pCMV-Tax). Transfection of pCMV-Tax inhibited E47-induced activation in a concentration-dependent manner (Fig. 1B). Thus, at the highest amount (500 ng) of the Tax vector, an 85% inhibition of E47 transcriptional activity was observed. A Western blot analysis of cell extracts showed that Tax expression did not inhibit E47 synthesis, but instead led to an increase of E47 expression, likely due to the transcriptional activation of the cytomegalovirus promoter by Tax. As underlined above, Tax has been shown to inhibit CBP/p300-mediated transcription by interfering with recruitment of these coactivators onto DNA of E-box elements (33, 38). Indeed, the cotransfection of a p300 vector was found to partially restore the basal E47-dependent activation (Fig. 1C). Collectively, these observations indicate that Tax represses the activity of the pT α promoter by inhibiting the transcriptional function of E2A proteins.

Tax expression inhibits pT α gene transcription in MOLT-4 cells. We then asked whether Tax would be able to act on pT α gene expression in T lymphocytes. In order to address this question, we elected to use the human MOLT-4 T-cell line, known to display a constitutive transcription of the pT α gene (35). We then expressed the Tax protein in these cells by gene transfer using lentiviral vectors in which Tax cDNA fused to the YFP marker gene was inserted downstream from the CMV promoter (10, 28). In addition, we also tested the pHR-TaxK88AYFP vector, which expresses the K88A Tax mutant unable to interact with CBP/p300 (19). First, the *trans*-activating or *trans*-inhibiting activity of these vectors was assessed in cotransfection assays by using either the LTR_{HTLV-1}-Luc plasmid containing the Tax-responsive elements within the HTLV-1 promoter or the EP180 construct containing E-box sequences. Luciferase assays performed 24 h after transfection clearly indicated that the Tax-YFP vector was as able as

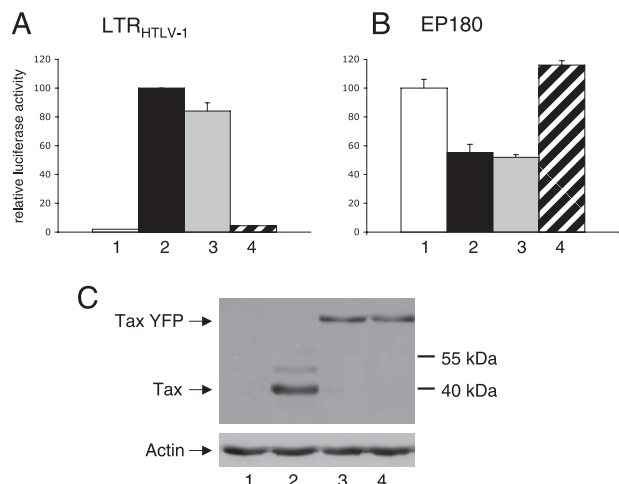


FIG. 2. Analysis of the transcriptional activity of TaxYFP fusion proteins. (A and B) HeLa cells were cotransfected either with 0.1 μ g of pLTR_{HTLV-1}-Luc (A) or 1 μ g of EP180 and 0.75 μ g of pCMV-E47 (B) reporter constructs together with a control plasmid (bar 1) or the pCMV-Tax (bar 2), pHR-TaxYFP (bar 3), or pHR-K88ATaxYFP (bar 4) plasmids (30 ng). The luciferase activities were normalized with tk-luc activities and are presented relative to those of cells transfected with pCMV-Tax (A) or with pCMV-E47 alone (B). The values shown are those obtained in triplicate from two experiments \pm standard deviations. (C) Western blot analysis of Tax expression. Equal amounts of whole-cell extracts of HeLa cells transfected as indicated above were analyzed by Western blotting using a polyclonal Tax antibody. The blots were reprobbed for actin as a loading control.

pCMV-Tax to activate or inhibit the transcription from the specific reporter construct (Fig. 2, compare lane 3 to lane 2). As expected, TaxK88AYFP was unable to activate transcription from the long terminal repeat (LTR) (Fig. 2A, lane 4), whereas it did not modify the transcription from EP180 (Fig. 2B, lane 4). Immunoblot assays performed to detect Tax confirmed the presence of a 40-kDa protein in cells transfected with pCMV-Tax and indicated that of a 65-kDa protein, corresponding to the Tax fusion protein in cells transfected with the TaxYFP or the TaxK88AYFP construct (Fig. 2C). Second, MOLT-4 cells were transduced either with the control pHR-YFP or the pHR-TaxYFP lentiviruses. Seven days after transduction, a flow cytometry analysis of YFP-expressing cells in one population of MOLT-4/YFP cells (MOI of 5) indicated that the percentage of YFP⁺ cells was 80.9%, whereas in two cultures of MOLT-4/TaxYFP transduced with an MOI of 2 and 5, that percentage was 21.5 and 58.5%, respectively (Fig. 3A). After RNAs were isolated and reverse transcribed, the cDNA samples were subjected to qPCR analysis, using primers specific for actin, pT α , and Tax. The pT α /actin ratio revealed that in MOLT-4/TaxYFP cells, the levels of pT α mRNAs were, respectively, 81 and 31% of those found in MOLT-4/YFP cells (Fig. 3A). Immunoprecipitation of cell extracts followed by immunoblot assays confirmed the presence of a 65-kDa protein in cells transduced by pHR-TaxYFP (Fig. 3B). Finally, a semiquantitative PCR analysis confirmed that in MOLT-4 cells transduced by the pHR-TaxYFP vector, the pT α gene is significantly less transcribed than in cells transduced by the pHR-YFP control vector (Fig. 3C, compare lane 4 to lane 1). These

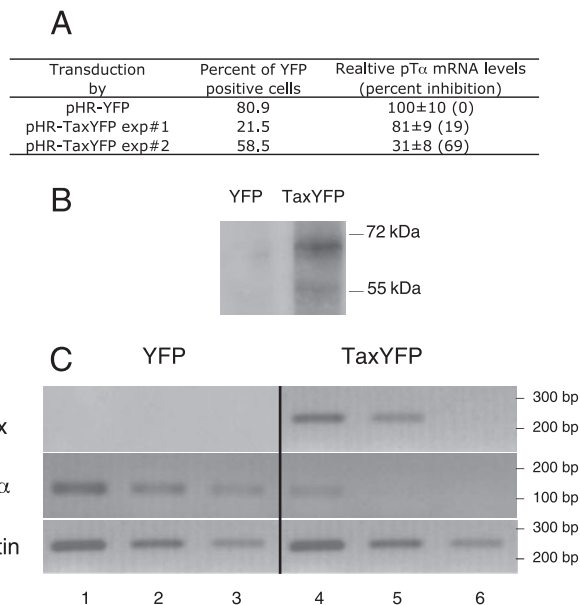


FIG. 3. Transcription of the pT α gene in TaxYFP- and control-transduced MOLT-4 cells. (A) Cells were transduced by either pHR-TaxYFP at an MOI of 2 (experiment 1) or 5 (experiment 2) or pHR-YFP at an MOI of 5. They were cultured for 7 days and analyzed by flow cytometry and by RT-qPCR for the determination of pT α mRNA levels. (B) Extracts of cells transduced by pHR-YFP or pHR-TaxYFP (experiment 2) were immunoprecipitated using a Tax antibody and then immunoblotted to determine the expression of the TaxYFP fusion protein. (C) cDNA samples from experiment 2 (undiluted, lanes 1 and 4; 1/5 diluted, lanes 2 and 5; 1/10 diluted, lanes 3 and 6) from pHR-YFP-transduced cells and from pHR-TaxYFP-transduced cells were subjected to semiquantitative PCR using primers specific for Tax, pT α , and actin.

observations thus clearly underline that the transcription of the pT α gene is inhibited in Tax-expressing MOLT-4 cells.

To extend these data, the YFP⁺ and YFP⁻ cells were then sorted from the MOLT-4/TaxYFP culture. The level of pT α gene transcription in these cells was analyzed and compared to that in GFP⁺- and GFP⁻-sorted MOLT-4 cells transduced by the Id3-IRES-GFP retroviral vector. As underlined in the introduction, the overexpression of Id3 in human CD34⁺ hematopoietic progenitor cells has been shown to correlate with the down-regulation of pT α gene expression (4). RT-qPCR analysis shows that the pT α gene expression was fourfold reduced in Tax-YFP⁺ cells compared to that detected in YFP⁻ cells sorted from this culture (Fig. 4). A similar pattern was observed in GFP⁺ cells sorted from the cells transduced with Id3-IRES-GFP vector. These data indicate that Tax as well as Id3 when overexpressed in MOLT-4 T cells decreases the transcription of the endogenous pT α gene. However, whereas Id3 is known to interfere with the transcriptional activity of E proteins by inhibiting their binding to E-box elements, the sequestration of CBP/p300 coactivators by Tax might explain the inhibitory action of this viral protein on the transcription of the pT α gene. To confirm this possibility, MOLT-4 T cells were transduced by the pHR-TaxK88AYFP vector, which expresses the K88A Tax mutant unable to interact with CBP/p300 (19). After isolation by fluorescence-activated cell sorting, qPCR analysis showed that no inhibition of the pT α gene expression

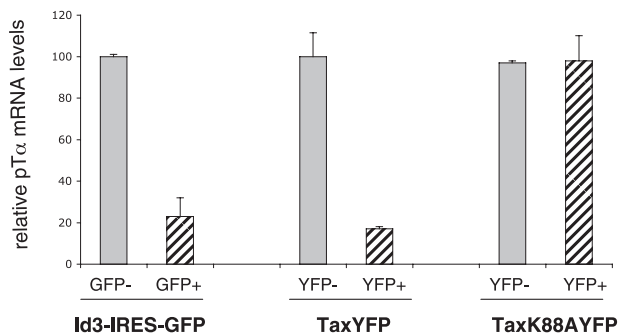


FIG. 4. Comparative analysis of the effects of Tax, TaxK88A, and Id3 on pT α gene transcription in MOLT-4 cells. Cells were transduced as described in Materials and Methods with either the Id3-IRES-GFP retrovirus or the pHR-TaxYFP or pHR-TaxK88AYFP lentiviruses. After 7 days of culture, GFP⁺ or YFP⁺ cells were sorted, and the cytoplasmic RNAs were isolated and reverse transcribed. The cDNA samples were subjected to qPCR using primers specific for pT α and normalized for the amount of cDNA, using actin as an internal control. Standard deviations are from at least two determinations performed in duplicate.

could be detected in TaxK88AYFP⁺-sorted cells compared to that detected in YFP⁻ cells sorted from these cultures. This observation extends the data presented above and supports that Tax inhibits pT α transcription by recruiting CBP/p300 coactivators.

Tax down-regulates pT α gene transcription in human immature thymocytes. We next asked whether Tax is modulating pT α gene expression in human immature thymocytes, in which, as underlined in the introduction, pT α transcription is up-regulated (5, 6, 43). After preparation of single-cell thymocyte suspensions, immature cells were negatively selected from the CD3⁻ population, as described in Materials and Methods. Their phenotypic analysis indicated the presence of a large majority of CD4 ISP (immature single positive) cells, besides CD34⁺ cells (Fig. 5A). Mature thymocytes, obtained after CD3-positive selection, were composed of cells expressing CD3 together with the CD4 and CD8 antigens (Fig. 5B). As expected, the level of pT α mRNAs assessed in both populations by RT-qPCR analysis was found to be fourfold higher in immature thymocytes than in mature thymocytes (Fig. 5C).

Because of the difficulty we encountered in transducing human immature thymocytes, we elected to transfect these cells by nucleofection with either the control pHR-YFP vector or the pHR-TaxYFP vector. Twenty-four hours after transfection, thymocytes were sorted into YFP⁺ and YFP⁻ populations. Then, mRNAs were isolated and analyzed by RT-PCR for the expression of pT α and of Tax. Comparable levels of pT α transcripts were observed in both populations sorted from control pHR-YFP-transfected cells (Fig. 6A). Whereas a similar amount of pT α transcripts was detected in the YFP⁻ cells sorted from thymocytes transfected with the pHR-TaxYFP vector, the pT α gene expression was highly reduced in the corresponding YFP⁺ thymocytes (Fig. 6B). Such a decrease was found to correlate with the level of Tax mRNAs, which were indeed detected in these thymocytes, but not in the YFP⁻ cells. To further quantify the decreased transcription of the pT α gene, we performed RT-qPCR assays, which revealed a 73% inhibition of the transcription of this gene in Tax-express-

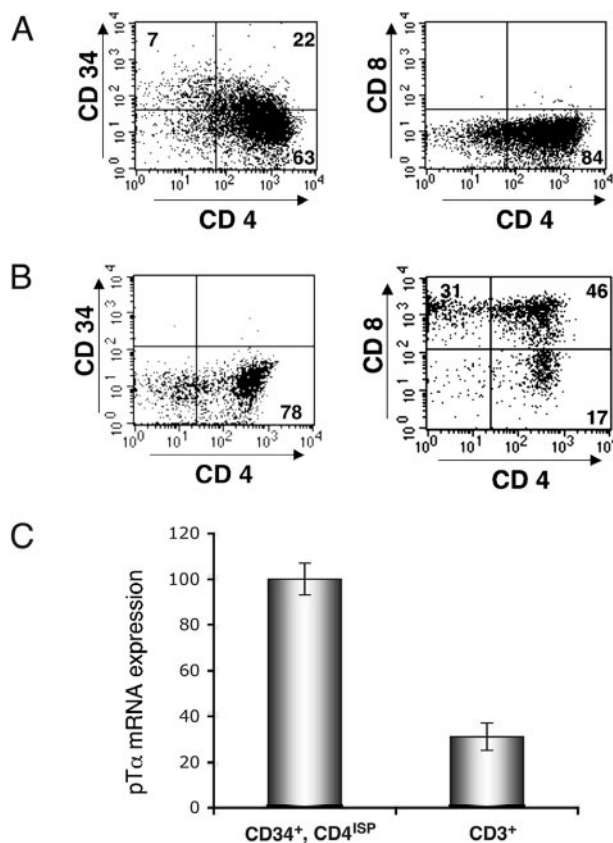


FIG. 5. Characterization of the immature and mature human thymocytes. (A) Two-color flow cytometry analysis of CD34⁺ and CD4 ISP cells recovered from human thymus. Single-cell suspensions were prepared and analyzed for the expression of the indicated surface antibodies. Numbers in the dot plots represent percentages of dots in each quadrant. (B) Two-color flow cytometry analysis of CD3⁺ thymocytes. (C) Quantification of pT α gene expression in immature and mature thymocytes by qPCR. The values for pT α mRNA expression relative to those of actin mRNAs were calculated from a standard curve of cDNA obtained from MOLT-4 cells.

ing thymocytes (Fig. 6C). These observations indicate that the transcription of the pT α gene is strongly repressed in Tax-expressing immature thymocytes. The amount of E47 mRNAs was next evaluated to exclude the possibility that Tax exerts an inhibitory effect on the transcription of this E2A gene. No difference could be observed in the level of E47 transcription in YFP-positive and YFP-negative thymocytes sorted after transfection with either the control vector or the TaxYFP vector (Fig. 6B). Collectively, these data indicate that the decreased transcription of the pT α gene in human immature thymocytes expressing Tax is likely correlated with the inhibition of the transcriptional activity of E2A proteins induced by this HTLV-1 regulatory protein.

DISCUSSION

Numerous studies devoted to the regulation of the transcription of the pT α gene during early $\alpha\beta$ T-cell development have underlined the importance of ubiquitous bHLH E2A proteins. Thus, the overexpression of E47, one of the two E2A proteins, and its binding to E-box sequences present in the promoter of

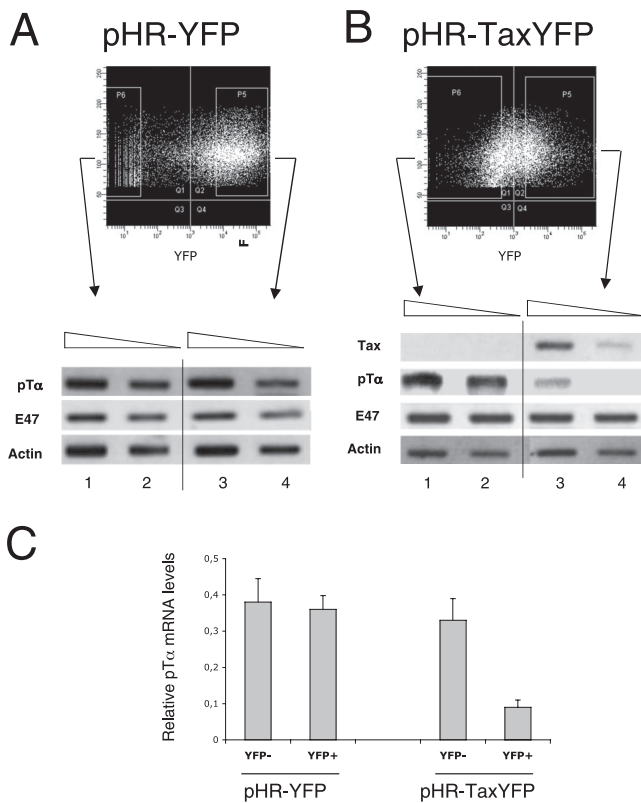


FIG. 6. Tax down-regulates pT α gene transcription in human immature thymocytes. Human immature (CD34⁺ CD3⁻ CD4⁺ ISP) thymocytes were isolated and nucleofected either with a pHR-YFP control vector (A) or with a pHR-TaxYFP vector (B). They were incubated in complete RPMI containing interleukin-7 and stem cell factor. Twenty-four hours later, the YFP⁻ and YFP⁺ cells were sorted from both cultures. The RNAs were isolated and reverse transcribed. Twofold-diluted cDNA samples from YFP⁻ cells (lanes 1 and 2) and from YFP⁺ cells (lanes 3 and 4) were subjected to PCR (A and B, lower panels) and to qPCR (C) using primers specific for the indicated genes.

the pT α gene resulted in increased transcription of this gene (27, 30, 40). In the present study, we first showed in transient-transfection experiments that the HTLV-1 Tax protein down-regulated the activation of the pT α promoter mediated by the ectopic expression of E47. We also noticed that expression of the coactivator p300 restored the activation of the pT α promoter. These observations are in line with previous results, showing that the interaction of Tax with p300 represses the transcriptional activity of bHLH proteins (33, 38). It has been reported that Tax binds to the KIX domain of p300, with which E2A interacts directly (2, 15, 33). Interestingly, we have observed that cotransfection of a plasmid encompassing this region is also able to restore the activation of the pT α promoter (data not shown). Under such conditions, Tax expression in lentivirally transduced MOLT-4 cells was found to correlate with a decreased transcription of the pT α gene. Furthermore, we report that a Tax mutant (K88A) unable to interact with CBP/p300 was found to be also unable to repress the transcription of pT α gene. We have finally looked for the activity of Tax on pT α transcription in human immature thymocytes (mainly CD4⁺ ISP), in which the pT α gene is very highly transcribed. We

observed that pT α expression was repressed and that the transcription of the E47 gene was not decreased in these cells when they were expressing Tax. This Tax-induced E2A protein silencing mechanism is reminiscent of that associated with the AML-ETO fusion protein that inhibits transcriptional activation by E proteins through stable interactions that preclude recruitment of the coactivators (47). This mechanism is therefore fundamentally distinct from that used by Id proteins which, through interactions with DNA binding regions of E2A proteins, block binding to the promoter (11, 12).

During early thymocyte development, the E2A proteins function as gatekeepers to arrest both differentiation and proliferation of thymocytes with defects in the assembly of the pre-TCR complex. Furthermore, they are endowed with a potential tumor-suppressing activity, as inactivation of E2A transcriptional activity is an essential and common step toward the development of T-cell malignancies. Thus, in mice, disruption of the E2A gene or inhibition of E2A proteins by Id proteins leads to abnormalities in the earliest stages of $\alpha\beta$ T-cell development and to varying degrees of reduced thymic cellularity (1, 11, 22, 23). Later in life, these E2A-deficient mice become prone to developing highly malignant T-cell lymphomas, most of them expressing either CD4 or CD8. These observations suggest that before the onset of T-cell lymphomas, genetic mutations or epigenetic alterations have accumulated in the surviving thymocytes that can undergo leukemic growth. Interestingly, the developmental stages of the neoplastic cells are beyond the initial developmental block.

By decreasing the expression of the pT α invariant chain, Tax is interfering with the assembly of the pre-TCR. As pre-TCR signals are required for the developmental transition from the double-negative to the double-positive stage, this complex is critical for further proliferation and differentiation. This first checkpoint in T-lymphocyte development (also referred to as the β -selection checkpoint) has been shown to begin in a small subset of immature thymocytes referred to as CD4⁺ ISP cells (mainly used in the present study). Then, β -selection continues in double-positive cells that up-regulate first the expression of CD8 α and, subsequently, that of CD8 β . Finally, the expression of GLUT1, the recently identified receptor for HTLVs, is restricted to a significant percentage of these immature thymocytes undergoing β -selection (26, 39). Interestingly, we have previously shown that these cells can be productively infected by HTLV-1 (25).

Consequently, the present study allows us to propose that Tax expression in immature thymocytes might lead to the emergence of pre-TCR⁻ Tax⁺ cells. The fate of these cells would therefore be dependent on the pleiotropic activities of this viral protein, which dysregulates the transcription of genes involved in cellular proliferation, cell cycle control, apoptosis, and rapid senescence, mainly through the constitutive activation of the NF- κ B pathway (14, 24, 41). Thus, these Tax-expressing cells might either undergo programmed cell death or survive and/or proliferate. Thymic hypocellularity was observed in transgenic mice expressing HTLV-1 pX regulatory proteins (among which is Tax) (29). In rabbits inoculated with HTLV-1-infected T cells, thymic atrophy in the presence of rapidly increasing thymic proviral load was observed and preceded the development of an acute ATL-like malignant lymphoproliferative disease (37). Thymic atrophy has also been

observed in 26 ATL patients who have undergone autopsy (18). Likewise, the frequent occurrence of opportunistic infections in ATL patients and in HTLV-1 carriers was found to correlate with a low number of naive T lymphocytes (45). Levels of T-cell receptor rearrangement excision circles, which are generated by DNA recombination during early T lymphopoiesis, were lower in HTLV-1-infected individuals than in uninfected levels, suggesting a suppressed production of T lymphocytes in HTLV-1-infected individuals.

Alternatively, the perturbation of thymocyte subset distributions in some SCID-hu mice inoculated with HTLV-1-infected CD34⁺ or T cells has suggested that the dysregulation of thymopoiesis may be a predisposing event in the development of leukemia (13). In addition, as exemplified with the E2A-deficient mice, the expression of Tax, by inactivating E2A proteins in immature thymocytes, might be followed by the emergence of a malignant lymphoproliferation. Thus, transgenic mice, in which Tax is expressed under the control of the Lck proximal promoter (which restricts transgene expression to developing thymocytes), after a long latency period develop lymphoma and leukemia with constitutive activation of NF- κ B (20). As a matter of fact, activation of this pathway is one of the main features of ATL cells, in which Tax expression is not or is rarely detected (14, 17). Furthermore, ATL cells are generally CD4⁺ T cells, although other less common phenotypes, such as CD4⁻ CD8⁻, CD8⁺, and CD4⁺ CD8⁺, have been observed (9, 21, 36, 44). We therefore propose that, in the early stages of the disease, Tax in human immature thymocytes would favor the survival, the proliferation, and the differentiation of some of these HTLV-1-infected cells, thus providing a substrate population for secondary mutations and/or further altered gene expression. Such a process would ultimately lead to the malignant growth of clones in which viral expression would be restricted. The leukemogenic signal triggered by Tax might thus be not only restricted to a critical stage of T-cell development but also linked to the silencing of E2A proteins together with the activation of the NF- κ B pathway.

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